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A progesterone metabolite stimulates the release of gonadotropin-releasing hormone from GT1-1 hypothalamic neurons via the γ-aminobutyric acid type A receptor

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ABSTRACT **The reduced progesterone metabolite tetrahydroprogesterone (3α-hydroxy-5α-pregnan-20-one; 3α,5α-THP) is a positive modulator of the γ-aminobutyric acid type A ($GABA_A$) receptor. Experiments performed *in vitro* with hypothalamic fragments have previously shown that GABA could modulate the release of gonadotropin-releasing hormone (GnRH). Using GT1-1 immortalized GnRH neurons, we investigated the role of $GABA_A$ receptor ligands, including 3α,5α-THP, on the release of GnRH. We first characterized the $GABA_A$ receptors expressed by these neurons. [^{3}H]Muscimol, but not [^{3}H]flunitrazepam, bound with high affinity to GT1-1 cell membranes (K_d = 10.9 ± 0.3 nM; B_{max} = 979 ± 12 fmol/mg of protein), and [^{3}H]muscimol binding was enhanced by 3α,5α-THP. mRNAs encoding the α_1 and β_3 subunits of the $GABA_A$ receptor were detected by the reverse transcriptase polymerase chain reaction. In agreement with binding data, the benzodiazepine-binding γ subunit mRNA was absent. GnRH release studies showed a dose-related stimulating action of muscimol. 3α,5α-THP not only modulated muscimol-induced secretion but also stimulated GnRH release when administered alone. Bicuculline and picrotoxin blocked the effects of 3α,5α-THP and muscimol. Finally, we observed that GT1-1 neurons convert progesterone to 3α,5α-THP. We propose that progesterone may increase the release of GnRH by a membrane mechanism, via its reduced metabolite 3α,5α-THP acting at the $GABA_A$ receptor.**

Some reduced metabolites of progesterone (PROG) have been shown to interact with the γ-aminobutyric acid type A ($GABA_A$) receptor (1, 2). In particular, the neurosteroid 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP), which has been called a neurosteroid because it can be formed from PROG by glial cells and neurons in primary cultures (reviewed in ref. 3), enhances the binding of several ligands of the $GABA_A$ receptor such as muscimol and flunitrazepam (1), decreases the binding of the convulsant *t*-butyl-bicyclophosphorothionate (4), and increases GABA-activated chloride conductance (5, 6). In addition, at high concentrations, 3α,5α-THP behaves as a $GABA_A$ agonist and stimulates Cl^- conductance and ^{36}Cl influx into synaptoneurosomes (1) in the absence of GABA.

Experiments performed *in vitro* with superfused arcuate nucleus-median eminence fragments (7, 8) have indicated that $GABA_A$ receptor agonists stimulate gonadotropin-releasing hormone (GnRH) release. Recently, Mellon *et al.* (9) have immortalized hypothalamic GnRH neurons by genetically targeted tumorigenesis in transgenic mice, GT1 cells and their sublines GT1-1, GT1-3, and GT1-7 express GnRH mRNA, generate GnRH pulses spontaneously (10, 11), and release GnRH in response to depolarization (12). Muscimol has been previously shown to stimulate the release of GnRH from GT1-1 cells (13, 14) and from GT1-7 cells (15). We now report that GT1-1 cells express $GABA_A$ receptors, release GnRH when exposed to the $GABA_A$ receptor agonists muscimol and 3α,5α-THP, and can synthesize 3α,5α-THP from PROG.

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METHODS

Cell Culture. GT1-1 cells (generously provided by R. Weiner and A. Choi, University of California, San Francisco) were cultured in 100-mm Petri dishes or six-well dishes (Nunc), coated with 3 μg of poly(L-ornithine) per ml (Sigma), in a medium comprising a 1:1 mixture of conditioned medium from mouse embryonic astrocytes in primary culture (16) and of Dulbecco's modified Eagle's medium (DMEM; GIBCO), supplemented with 10% fetal calf serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml (conditioned DMEM: C-DMEM). The use of conditioned medium is essential for the growth of GT1-1 neurons. When cells reached 90% confluency, C-DMEM was replaced by a serum-free medium (Opti-MEM, GIBCO) for 24 hr.

GnRH Release. On the day of the experiment, confluent GT1-1 cells, replated on six-well dishes (4.6 ± 0.7 × 10^6 cells per well), were washed with 2 ml of Locke's medium (mM concentrations: NaCl, 154; KCl, 5.6; $CaCl_2$, 2.2; $MgCl_2$, 1; $NaHCO_3$, 6; glucose, 10; Hepes, 2; pH 7.4) supplemented with 20 μM bacitracin (Sigma). They were then incubated at 37°C for 30 min in 1 ml of Locke's/bacitracin medium with or without muscimol and/or 3α,5α-THP at various concentrations (three wells per condition). At the end of the incubation period, media were stored at −20°C until radioimmunoassayed for GnRH.

GnRH RIA. The concentration of GnRH released into the medium (1 ml per well) was measured by RIA in triplicate, with a charcoal precipitation method, modified from Nett *et al.* (17), using ^{125}I-labeled GnRH (^{125}I-GnRH) (2000 Ci/mmol; 1 Ci = 37 GBq; Amersham), nonlabeled GnRH (Fluka) as the standard, and the rabbit polyclonal antibody R1245 (obtained from T. Nett, Colorado State University, Fort Collins), which is specific for the decapeptide. Briefly, 100 μl of antibody (1:76,800 final dilution) was added to each tube already containing 200 μl of sample (or nonlabeled GnRH for the standard curves) and 200 μl of GnRH assay buffer (mM concentrations: NaCl, 145; EDTA, 25; NaH_2PO_4, 3.3; Na_2HPO_4, 6.7) supplemented with merthiolate (10 mg/liter, Sigma), and gelatin (1 g/liter, pH 7.4). After 2 hr at 4°C, 100 μl of ^{125}I-GnRH (12,000 cpm) was added to all tubes and incubation was continued for an additional 24 hr. Seven hundred fifty microliters of a charcoal/dextran suspension (2.5 g/liter and 0.25 g/liter, respectively, in Ca^{2+}- and Mg^{2+}-free

Abbreviations: GnRH, gonadotropin-releasing hormone; $GABA_A$ receptor, γ-aminobutyric acid type A receptor; 3α,5α-THP, 3α-hydroxy-5α-pregnan-20-one; 5α-DHP, 5α-pregnane-3,20-dione; RT-PCR, reverse transcriptase polymerase chain reaction; LH, luteinizing hormone; PROG, progesterone.

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phosphate-buffered saline) was then added, the tubes were centrifuged at 4000 × g for 10 min, and the supernatants were counted for radioactivity. All samples from an experiment were analyzed in the same assay. The lower limit of detection was 4 pg/ml.

[^{3}H]Muscimol and [^{3}H]Flunitrazepam Binding. GT1-1 cells were homogenized in 10 vol of ice-cold 10 mM potassium phosphate buffer (KPB; pH 7.4) and centrifuged at 48,000 × g for 20 min. The pellets were resuspended in 10 mM KPB and stored at −20°C for up to 1 month. When needed, membranes were thawed, immediately suspended in 10 mM KPB, centrifuged at 48,000 × g for 20 min, and resuspended in 50 mM KPB supplemented with 100 mM KCl (pH 7.4) at a protein concentration of 1.2 mg/ml. Membranes (200 μl; 240 μg of protein) were preincubated at 4°C in the presence or absence of increasing concentrations of 3α,5α-THP (Sigma) in a total volume of 300 μl. The steroid was dissolved in 0.5% dimethyl sulfoxide. Fifteen minutes later, 200 μl of [^{3}H]muscimol (26 Ci/mmol; Amersham) was added (final volume, 500 μl). After 60 min, samples were filtered through Whatman GF/B glass fiber filters under vacuum. The filters were washed and counted for radioactivity in 10 ml of Picofluor 15 scintillation fluid (Packard Instruments, Meriden, CT). For subtraction of nonspecific binding, parallel incubations were performed in the presence of unlabeled muscimol at a concentration 10^3-fold larger than that of the radioactive ligand. [^{3}H]Flunitrazepam binding studies were performed under the same conditions, in the presence of 20 nM [^{3}H]flunitrazepam (85 Ci/mmol; Amersham) with or without 20 μM unlabeled flunitrazepam.

$GABA_A$ Receptor Subunit mRNAs. Total cellular RNA was isolated from GT1-1 cells or from rat brain by the acid phenol method (18). We used the reverse transcriptase polymerase chain reaction (RT-PCR) to detect the expression of individual $GABA_A$ receptor subunits. The sequences of subunit-specific 24-mer primer pairs were kindly provided by D. Grayson (Georgetown University, Washington, DC) and corresponded to unique sequences of the intracellular loop of the respective subunits. The nucleotide sequences amplified were as follows: α_1, 1178–1482; α_2, 1534–1867; α_3, 1432–1783; α_4, 1315–1709; α_5, 1188–1526; α_6, 1028–1376; β_1, 1190–1531; β_2, 1201–1518; β_3, 1199–1554; γ_1, 1098–1458; γ_2, 1156–1492; γ_3, 1099–1430; δ, 1097–1430. Internal control 68-kDa neurofilament protein primers were also added to each reaction mixture. First strand cDNA synthesis was performed by the incubation of 500 ng of random hexamer primers (New England Biolabs) with 4 μg of total RNA, 20 units of an RNase inhibitor (Promega), 1 mM of each nucleotide triphosphate (Pharmacia), and 800 units of Moloney murine leukemia virus reverse transcriptase (New England Biolabs) in a total volume of 200 μl. After 2 hr at 37°C, the cDNA template/RNA mixture was ethanol-precipitated. The PCRs of 100 μl contained cDNA template, 10 μl of 10× enzyme reaction buffer (New England Biolabs), 200 μM of each nucleotide triphosphate, 100 pmol of each forward and reverse primers, and 2 units of $Vent_R$ DNA polymerase (New England Biolabs). Each amplification cycle (30 cycles) consisted of denaturing at 94°C for 45 sec, annealing at 62°C for 1 min, and extending at 72°C for 1 min with a final extension time of 5 min. The PCR products (20 μl of each) were analyzed by electrophoresis on 1.4% agarose gels containing ethidium bromide (0.5 μg/ml).

[^{14}C]PROG Metabolism. GT1-1 cells were incubated at 37°C for 24 hr in 10 ml of serum-free Opti-MEM supplemented with 100 nM [^{14}C]PROG (57.2 mCi/mmol, Amersham). Media were collected and extracted three times with 2 vol of ethyl acetate/isooctane (1:1, vol/vol), and the combined extracts were taken to dryness in a vacuum centrifuge (Speed-Vac concentrator, Savant). Thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) were performed as described (19). Briefly, TLC was on silica gel 60 F254 TLC plates (Merck) in the solvent system chloroform/ethyl acetate (4:1, vol/vol). Reference compounds—5α-pregnane-3,20-dione (5α-DHP, Roussel-UCLAF), 3α, 5α-THP (Sigma), and PROG (IBF, Villeneuve-la-Garenne, France)—were run on separate lanes. Their R_f values were 5α-DHP, 0.72; 3α,5α-THP, 0.49; and PROG, 0.61. Radioactive steroids were analyzed and quantified with an automatic TLC linear analyzer (Multitrace Master LB-285, Berthold, Nashua, NH). The corresponding radioactive areas were scraped off and eluted with 5 ml of ethyl acetate. Reverse-phase HPLC was performed on a 5-μm C_{18} ODS column using a linear gradient of 60–80% methanol in 30 min and fractions (1 ml/min) were collected. The retention times of reference 3α,5α-THP and 5α-DHP were 23–25 min and 7–9 min, respectively. Radioactivity recovered from TLC and HPLC fractions was counted. The amounts of PROG metabolites produced by GT1-1 cells were expressed in pmol/μg of DNA (≈35 μg of DNA per dish). DNA was measured by the mithramycin fluorescence technique (20).

Statistics. All results were expressed as mean ± SEM. Comparisons of data were carried out by Student's t test.

RESULTS

Stimulation of GnRH Release by Muscimol and by 3α,5α-THP. The spontaneous release of GnRH by 4.6 ± 0.7 × 10^6 cells over 30 min was 418 ± 22 pg/ml (1 ml per well). The $GABA_A$ agonist muscimol (0.5–10 μM) caused a concentration-related increase of GnRH release, up to a 3-fold increase (1300 ± 56 pg/ml) over basal levels (Fig. 1). The neurosteroid 3α,5α-THP also greatly enhanced GnRH secretion in the absence of muscimol. The effect of 3α,5α-THP was rapid: a significant stimulation was already detectable after 3 min (basal release, 310 ± 7 pg/ml; 10 μM 3α,5α-THP, 538 ± 18 pg/ml); however, as mentioned above, the release of GnRH was routinely measured after 30 min. 3α,5α-THP-induced stimulation of GnRH secretion was concentration-dependent and reached the same maximum value as muscimol-evoked release (1253 ± 53 pg/ml) at a steroid concentration 10-fold lower (1 μM) than that of muscimol (Fig. 1). Compared to the effect of 1 μM 3α,5α-THP, 10 μM 3α,5α-THP induced a significantly smaller stimulation of GnRH secretion.

Modulation of Muscimol-Induced GnRH Secretion by 3α,5α-THP. Besides its direct stimulation of GnRH release, 3α,5α-THP modulated muscimol-evoked GnRH secretion. The resulting effect depended on the respective concentrations of both 3α,5α-THP and muscimol. When GT1-1 cells were

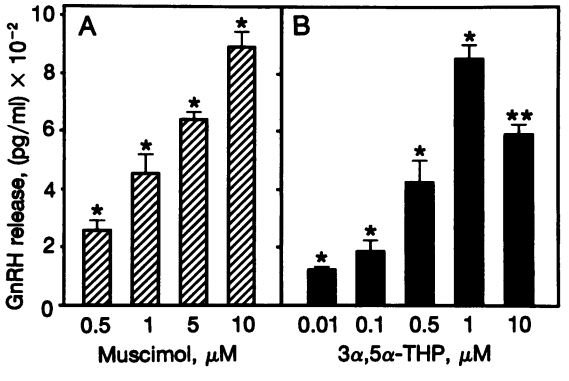

FIG. 1. Muscimol or 3α,5α-THP stimulates the release of GnRH. GT1-1 cells (4.6 ± 0.7 × 10^6 per well) were incubated for 30 min in 1 ml of Locke's medium without or with increasing concentrations of either muscimol (*A*) or 3α,5α-THP (*B*). The spontaneous release of GnRH (418 ± 22 pg/ml) has been subtracted. Results express the mean ± SEM of three or four experiments performed in triplicate. *, $P < 0.001$ vs. spontaneous release; **, $P < 0.001$ vs. release induced by 1 μM 3α,5α-THP.

coincubated with a low dose of muscimol (0.5 μM) and increasing concentrations of 3α,5α-THP (0.01–10 μM), the effects of muscimol and 3α,5α-THP were additive (Fig. 2). When a high concentration of muscimol was used (10 μM), coincubation with low concentrations of 3α,5α-THP (0.01–0.1 μM) did not further increase muscimol-induced stimulation of GnRH release. However, higher concentrations of 3α,5α-THP (1 and 10 μM) significantly inhibited the stimulatory action of 10 μM muscimol (Fig. 3).

Involvement of the $GABA_A$ Receptor. As expected for a $GABA_A$ receptor ligand, the effect of muscimol on GnRH release was abolished by the specific $GABA_A$ antagonist bicuculline (100 μM) and by the chloride-channel blocker picrotoxin (100 μM) (Fig. 4). 3α,5α-THP-induced secretion was also suppressed by bicuculline and picrotoxin, confirming the involvement of a $GABA_A$ receptor in the action of the steroid. In addition, neither bicuculline nor picrotoxin modified the basal release of GnRH, thus suggesting an absence of secretion of GABA by GT1-1 cells.

Binding Studies. Membranes of GT1-1 cells bound the $GABA_A$ agonist [^{3}H]muscimol in a concentration-related manner. Specific binding represented 90% of total binding. Scatchard analysis revealed a single category of high-affinity binding sites, with a dissociation constant (K_d) of 10.9 ± 0.3 nM and a maximal concentration of binding sites (B_{max}) of 979 ± 12 fmol/mg of protein. Moreover, when GT1-1 cell membranes were incubated with 3α,5α-THP, the binding of [^{3}H]muscimol was enhanced in a concentration-dependent manner, leading to a maximal potentiation of 48% (Table 1). In contrast, no significant binding of the benzodiazepine [^{3}H]flunitrazepam (20 nM) was observed, and flunitrazepam did not affect [^{3}H]muscimol binding (data not shown). These results suggest that $GABA_A$ receptors are present in GT1-1 neurons but that they lack benzodiazepine binding sites.

Expression of $GABA_A$ Receptor Subunit mRNAs. We used the RT-PCR amplification technique to detect the presence of mRNAs encoding the α_{1-6}, β_{1-3}, γ_{1-3}, and δ $GABA_A$ receptor subunits. The efficiency of RT-PCR was checked in each sample by including neurofilament protein primers. After 30 cycles, the RT-PCR products were analyzed by electrophoresis on agarose gel (Fig. 5). Only two bands corresponding to α_1 and β_3 subunit mRNAs were detected in GT1-1 cells, whereas whole rat brain expressed mRNAs corresponding to all tested subunits.

Metabolism of PROG in GT1-1 Hypothalamic Neurons. Cells were incubated with [^{14}C]PROG and the steroids present in the incubation medium were separated by TLC. Besides unmetabolized PROG, radioactive spots with the R_f values of dihydroprogesterone (5α-DHP) and 3α,5α-THP were detected on autoradiograms and scanned (Fig. 6). Very polar radioactive metabolites were also detected but were not further characterized. The amounts of metabolites formed in the experimental conditions reported in *Methods* were 8.2 and 1.1 pmol/μg of DNA for 5α-DHP and 3α,5α-THP, respectively. The radioactive area corresponding to 3α,5α-THP was eluted and further characterized by HPLC, with a retention time of 23 min.

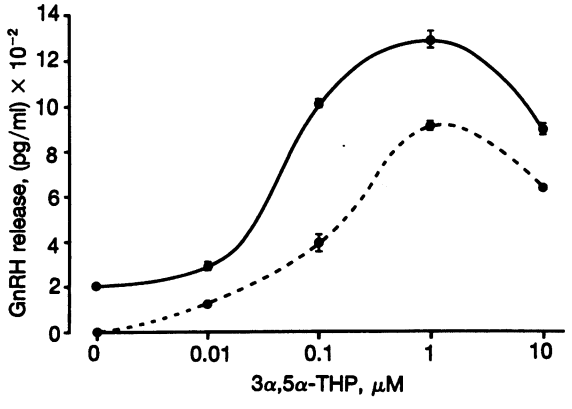

FIG. 2. 3α,5α-THP potentiates the release of GnRH induced by a low concentration of muscimol. Neurons were incubated with muscimol (0.5 μM) and/or increasing concentrations of 3α,5α-THP. —, Muscimol plus 3α,5α-THP; - -, 3α,5α-THP alone. Muscimol-induced GnRH release was 200 ± 12 pg/ml over the basal value. Basal release (458 ± 6 pg/ml) was subtracted from all values. Results express the mean ± SEM of three experiments performed in triplicate.

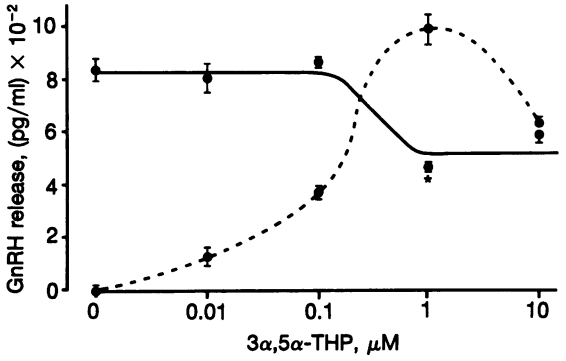

FIG. 3. Effect of 3α,5α-THP on the release of GnRH evoked by a high concentration of muscimol. GT1-1 cells were incubated with muscimol (10 μM) and/or increasing doses of 3α,5α-THP. —, Muscimol plus 3α,5α-THP; - -, 3α,5α-THP alone. Muscimol-evoked GnRH release was 852 ± 42 pg/ml over the basal value. Basal release (438 ± 18 pg/ml) was subtracted from all values. Data are the mean ± SEM of four experiments performed in triplicate. *, $P < 0.001$ vs. muscimol-induced release.

DISCUSSION

GABA has been reported to stimulate and inhibit luteinizing hormone (LH) secretion *in vivo*, depending on the dose and the hormonal status of the animal (21). At the hypothalamic level, experiments performed *in vitro* with arcuate nucleus-median eminence fragments have shown that muscimol stimulated while baclofen inhibited GnRH secretion (7, 8). In those preparations, it was unclear whether the effects of GABA

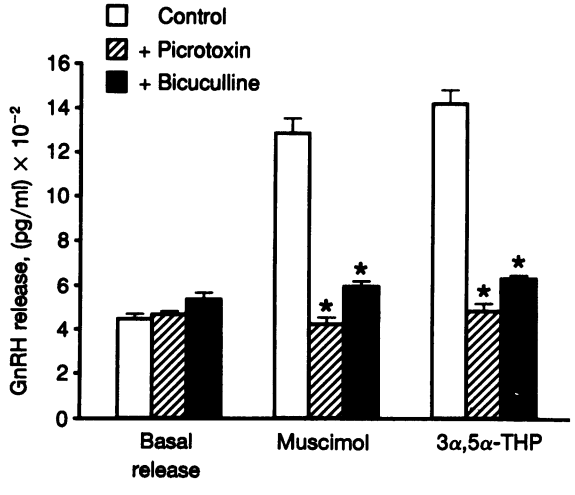

FIG. 4. Effects of bicuculline and picrotoxin on GnRH release. GT1-1 cells were incubated with the $GABA_A$ channel blocker picrotoxin (100 μM) or the specific $GABA_A$ receptor antagonist bicuculline (100 μM), alone or with either muscimol (10 μM) or 3α,5α-THP (1 μM). Data are the mean ± SEM of three experiments performed in triplicate. *, $P < 0.001$ vs. the respective control.

Table 1. Potentiation of [^{3}H]muscimol binding by 3α,5α-THP

Parameter	3α,5α-THP, μM				
	0	0.01	0.1	1	10
Muscimol bound (fmol/mg of protein)	319 ± 7	334 ± 6	363 ± 8	424 ± 8	472 ± 12
Potentiation of muscimol binding (%)	—	5	14	33	48

Increasing concentrations of 3α,5α-THP (0.01–10 μM) enhanced the binding of 5 nM [^{3}H]muscimol to membrane preparations of GT1-1 cells. Results are expressed as the mean ± SEM of three experiments performed in triplicate.

directly implicated GnRH neurons or whether they involved surrounding neurons. In the present study performed on GT1-1 immortalized neurons, we show a direct stimulation of GnRH secretion by the $GABA_A$ receptor agonist muscimol, in agreement with the data reported by others on GT1-7 cells (15), and superfused GT1-1 cells (14).

We report that GT1-1 hypothalamic neurons express $GABA_A$ receptors and this conclusion is supported by [^{3}H]muscimol binding studies. The equilibrium binding constants measured (K_d = 10.9 nM; B_{max} = 979 fmol/mg of protein) are within the range reported for crude brain membranes and for $GABA_A$ receptors expressed in transfected cells (22, 23). [^{3}H]Muscimol binding to GT1-1 cell membranes is not increased by the benzodiazepine flunitrazepam and there is no binding of [^{3}H]flunitrazepam. Pritchett *et al.* (23) previously showed that the γ subunit is needed for an interaction of benzodiazepines with the $GABA_A$ receptor. As expected from binding studies, we did not detect by RT-PCR any mRNA encoding a γ subunit and the only mRNAs that are amplified in GT1-1 cells correspond to the α_1 and β_3 subunits. The absence of γ subunits has been reported by others with GT1-7 cells (15, 24). However, Hales *et al.* (24) found a third $GABA_A$ subunit mRNA (β_1) in these cells, whereas Favit *et al.* (15), by Northern blot analysis, detected transcripts for only one subunit, namely β_3. The diversity of the subunits, numbering 16 at present (25), and the complexity of their combinations explain the great functional heterogeneity of $GABA_A$ receptors. We do not know whether the subunit composition found in GT1-1 cells is similar to the *in vivo* arrangement in GnRH neurons. *In situ* hybridization studies performed in the rat medial preoptic area have shown a preferential distribution of α_2, β_3, and γ_1 mRNAs, but α_1 was also detected in small amounts (26). Moreover, dual-label *in situ* hybridization histochemistry has

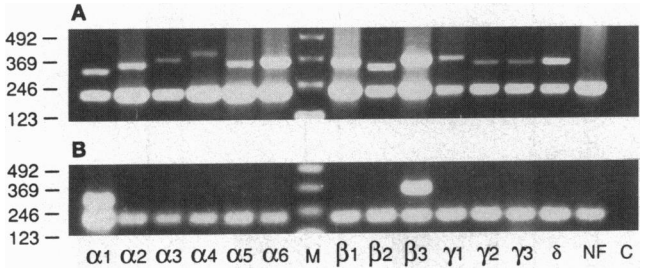

FIG. 5. RT-PCR amplification of $GABA_A$ receptor subunits expressed in GT1-1 cells. The two panels are from a set of reactions using RNAs extracted from either rat brain (*A*) or GT1-1 cells (*B*) as templates. Twenty microliters of amplification reaction mixture was submitted to electrophoresis on 1.4% agarose gels. The amplified fragments were specific for a single $GABA_A$ subunit (α_{1-6}, β_{1-3}, γ_{1-3}, and δ) or of control neurofilament (NF) protein 68. Lane NF, primers for NF protein alone; lane C, primers for NF without cDNA template; lane M, size marker (123-bp DNA ladder). Sequences amplified were as follows (bp): α_1 (304), α_2 (333), α_3 (351), α_4 (394), α_5 (338), α_6 (348), β_1 (341), β_2 (317), β_3 (355), γ_1 (360), γ_2 (336), γ_3 (331), δ (333).

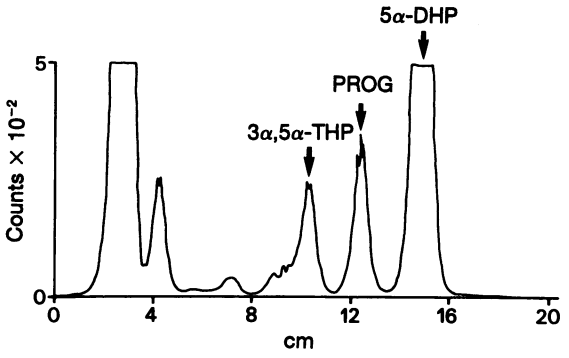

FIG. 6. Metabolism of [^{14}C]PROG. GT1-1 cells (35 × 10^6 cells per dish) were incubated for 24 hr with 100 nM [^{14}C]PROG. Steroids were extracted from the media and submitted to TLC. Radioactivity was measured with an automatic TLC linear analyzer.

conclusively demonstrated the predominant presence of the α_1 and β_3 subunits in ≈75% of rat GnRH neurons (27). This double-labeling technique has not been applied to the detection of γ subunit mRNAs; therefore the subtype and even the presence of γ subunits in GnRH neurons are unknown. To our knowledge, the absence of γ subunits observed in GT1 cells has not been reported for other brain cells, with the exception of a few cell lines (28, 29).

In GT1-1 cells expressing α_1 and β_3 subunits, we show that the neurosteroid 3α,5α-THP interacts with $GABA_A$ receptors since it enhances the binding of [^{3}H]muscimol. Previous studies, performed on transfected cells or *Xenopus laevis* oocytes injected with $GABA_A$ mRNAs, have been directed at identifying the subunits required for an interaction of 3α,5α-THP with the $GABA_A$ receptors. It was clearly demonstrated that 3α,5α-THP could modulate $GABA_A$ receptors in the absence of γ subunit (6, 30). A potentiation of GABA-activated currents was even observed in cells expressing homomeric β_1 receptors (6). When combinations of α and β or α, β, and γ subunits were used, the β subtype did not seem to influence 3α,5α-THP-induced modulation (31). The nature of the α subunit subtype was more critical. The best potentiation of GABA currents by 3α,5α-THP was obtained in oocytes expressing the α_1 and β_1 and α_1, β_1, and γ_1 combinations of bovine subunits (30), whereas the best effect of 3α,5α-THP on [^{3}H]muscimol or [^{3}H]flunitrazepam binding was obtained in cells transfected with α_3 and β_1 or α_3, β_1, and γ_2 rat subunit cDNAs, respectively (32, 33).

The present experiments show that 3α,5α-THP modulates the stimulation of GnRH release induced by muscimol. At all concentrations used, 3α,5α-THP increases the effect of a low dose of muscimol. However, when the release of GnRH is evoked by a high dose of muscimol, it is not enhanced by 3α,5α-THP, which even becomes inhibitory at concentrations ≥1 μM, suggesting receptor desensitization (25).

In addition to the modulation of the effect of muscimol, we clearly show a stimulating action of 3α,5α-THP on GnRH release in the absence of muscimol. 3α,5α-THP-induced stimulation of GnRH secretion is dose-related, biphasic (reaching its maximum at 1 μM and decreasing at higher concentrations), and rapidly observed (3 min). Effects of 3α,5α-THP on gene expression, after oxidation into 5α-DHP, which could activate the PROG receptor, have been already described in the brain (34). However, in our experiments, the rapid time course of 3α,5α-THP action renders such mechanism unlikely. In addition, since bicuculline and picrotoxin abolish 3α,5α-THP-induced GnRH secretion, $GABA_A$ receptors are clearly involved.

An increasing body of evidence assigns an important role to PROG in the preovulatory gonadotropin surge (35). Besides its direct regulatory effects on pituitary cells, PROG appears

to act at the hypothalamic level (36, 37) via the modulation of gonadotropin-releasing hormone synthesis and release. Indeed, PROG has been shown to facilitate GnRH release both *in vitro* and *in vivo* (38–40). One mechanism whereby PROG modulates GnRH secretion involves a regulation of gene expression, since PROG increases GnRH mRNA levels in the medial basal hypothalamus and preoptic area (37). However, PROG receptors have not been detected in GnRH secreting cells (41, 42). Since they are found in neighboring neurons, an indirect control of GnRH neurons via neuropeptides and/or neurotransmitters (43) was also suggested.

Other experiments performed on hypothalamic fragments with either progesterone-3-carboxymethyloxime bovine serum albumin (44) or 3β-hydroxy-5β-pregnan-20-one (45) have suggested a membrane site of action. The exposure time required was 1–2 hr. The target cells remained unknown. At any rate, $GABA_A$ receptors were not involved since none of these steroids does modulate $GABA_A$ receptors (2, 4).

The results we have obtained with GT1-1 neurons are consistent with the increase of serum LH observed by others after *in vivo* administration of 3α,5α-THP to ovariectomized rats (46, 47). Brann *et al.* (47) reported that picrotoxin, injected 30 min prior to the steroid, abolished the stimulation of LH release (47). Although these authors did not exclude a hypothalamic mediation of 3α,5α-THP action by $GABA_A$ receptors, they favored a direct regulation of gonadotropin secretion by the pituitary.

In conclusion, the present experiments performed with GT1-1 cells show that 3α,5α-THP, which may be synthesized by GnRH neurons, or by surrounding neurons and/or glial cells (3) or by peripheral organs (48), can directly and rapidly stimulate hypothalamic neurons to secrete GnRH by interacting with their $GABA_A$ receptors.

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